EUROPEAN PATENT APPLICATION

⑦ Applicant: BATTELLE MEMORIAL INSTITUTE
7 route de Drize
CH-1227 Carouge/Genève(CH)

㉒ Inventor: Myers, Andrew
19, Grand 'Rue
CH-1204 Geneve(CH)
Inventor: Bichon, Daniel
Chez Les Gris Groisy
F-74570 Thorens-Gilleres(FR)

㉔ Representative: Dousse, Blasco et al
7, route de Drize
CH-1227 Carouge/Genève(CH)

㊹ Advanced anticancer therapy and cytotoxic medicaments for its implementation.

㊲ A therapeutic composition comprising a chemical conjugate including a first moiety, other than an immunoglobulin or fragment thereof, such as epidermal growth factor, which preferentially binds to a tumor cell, and is internalized by the cell, and a second moiety linked to the first moiety, and comprising a biodegradable polymeric carrier, such as polyglutamic acid, to which one or more cytotoxic molecules, for instance, daunomycin, are attached. The degradation of the carrier by intracellular enzymes releases a cytotoxic agent, resulting in selective destruction of the tumor cells.

FIG. 2a   FIG. 2b

EP 0 259 904 A1

CYTOTOXIC DRUGS AND THEIR DELIVERY TO TUMOR CELLS

BACKGROUND OF THE INVENTION

Field of the Invention

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The present invention deals with novel chemical conjugates for the selective delivery of cytotoxic drugs to tumor cells, and with methods of destroying tumor cells using such conjugates.

10   Information Disclosure Statement

It is well known that current cancer therapy involves the use of antimiotic drugs such as adriamycin, vincristine, cisplatin, daunomycin and methothrexate, which all have strong undesirable side-effects on the normal cells of the patient. It is therefore important that the activity of antitumor drugs be specifically

15 directed to the malignant cells and have little toxic effect on the normal cells.

One approach to selectively delivering cytotoxic agents to tumor cells requires use of antibodies which preferentially bind tumor-associated or tumor-specific antigens such as alpha fetoprotein. The antibody may be radiolabeled, or it may be conjugated to toxins such as hematoporphyrin, abrin, ricin, diphtheria toxin, Pseudomonas exotoxin, gelonin or to the above-mentioned antimitotic drugs. For reviews see K. Sikora et

20 al., (1984), Br. Med. Bull., 40, 233-9; and P.C. Thorpe, et al., (1985), in Monoclonal Antibodies B4, Biological & Clinical Applications. Further, Pastan, US 4,545,985 discloses the binding of Pseudomonas exotoxin to antibodies, for instance antibodies to specific human cell receptors such as the transferrin receptor.

There are a number of disadvantages in the use of antibodies as targeting agents, as described by I. Pastan, et al., (1986), Cell, 47, 641-648. First, the internalization of anitbody conjugates into cells is highly

25 variable, depending on the antibody and the cell. Second, the antibody may itself be antigenic and stimulate an immune response in the patient, which would limit the effectiveness of the conjugate. Third, the antibody may bind to normal cells, which do not have the antigen recognized by the antibody, by means of Fc receptors that occur on many nontumor cells and can react with many antibodies. Fourth, it is difficult to attach a large number of drug molecules to an antibody without adversely affecting its antigen-binding

30 activity. [Hurwitz, et al., (1975), Cancer Res., 35 , 1175-1181]. Finally, the high molecular weight of antibodies reduces their ability to penetrate into tumors between the cells [See Delabye, et al., (1986), J. Clin. Invest. 77: 301-11; Buchegger. et al.)(1983), J. Exp. Med 158: 413-27; Buchegger et al. (1986), Cancer,58, 655-61.]

Another approach is to attach the drug to a polyaminoacid carrier which reduces its cytotoxic action in

35 normal cells. This carrier may bear a large number of drug molecules. In the drug-carrier complex, the amino acid composition or sequence is intended to provide preferential release of the cytotoxin at tumor cells by means of degradative enzymes known to be present in elevated concentrations in tumor cells. For example, Zunino, et al., (1982), Int. J. Cancer 30, 465-70, reports on the antitumor activity of daunorubicin linked to poly-L-aspartic acid, and Kato, et al., (1984), Cancer Res. 44, 25, describes antitumor activity of

40 cytosine arabinoside conjugated with polyglutamic acid. W.A.R. van Heeswijk, et al., (1985), J. Controlled Release 1, 301-315, and Hoes et al., (1985), J. Controlled Release 2, 205-213, describe additional conjugates of polyglutamic acid backbones with peptide spacers to which adriamycin is covalently coupled.

The combination of a tumor-directed antibody and a degradable polyaminoacid carrier with a cytotoxic drug has also been described. For example, Kato, et al., (1984), J. Medicinal Chem. 27, 1602-1607, reports

45 conjugation of daunomycin (DM) to polyglutamic acid (PGA) and coupling the resulting cytotoxic polymer with rat alpha fetoprotein (AFP) antibody. Upon trial, the cytotoxic acitivity of the resulting anti-AFP-PGA-DM conjugate was shown to be more effective than nIg (a control antibody), anti-AFP, unconjugated DM, PGA-DM or nIg-PGA-DM.

Similarly, EP-A 112,720 (Teijin) discloses a conjugate comprising an immunoglobulin capable of binding

50 selectively to a particular antigen possessed by a cell to be killed, a polymer carrier and a cytotoxic substance linked thereto, for instance p-(N,N-bis(2-Chloroethyl))-phenylenediamine, melphalan, 1-(beta-D-arabino-furanosyl) cytosine and its phosphate, m thotrexate, actinomycin D, mitomycin C and the like, while Runge, US 4,485,093 discloses an immunotoxin conjugate for treating malignant diseas s, which consists of arsanilic acid and tumor specific antibody covalently bound to a polyglutamic acid back-bon .

Unfortunately, the use of a polyaminoacid carrier does not solve all of the problems associated with the use of immunotoxins. In addition to the problems related to using antibodies directly conjugated to cytotoxins, addition of such carriers to antibodies, or even smaller Fab fragments, may undesirably further reduce their ability to penetrate tumors efficiently.

Pastan, US 4,545,985 suggested conjugating Pseudomonas exotoxin (PE) to a variety of peptides, proteins and growth factors that react with specific receptors on cells, including sarcoma growth factors, melanocyte stimulating hormone, somatostatin, glucagon, insulin, transferrin, low density lipoprotein (LDL), calcitonin, alpha-2-macroglobulin and lysine bradykinin. Pastan constructed a conjugate of Pseudomonas exotoxin (PE) and a peptide hormone isolated from mice, Epidermal Growth Factor (EGF), by introducing thiol groups into each and then linking the two using a disulfide exchange reaction. The conjugate was toxic to KB tumor cells, but nude mice injected with the conjugate died of liver failure. The coupling destroyed the toxin's ability to bind to its own receptor, so toxicity was mediated by the EGF receptors of the liver cells.

N. Schimizu, et al., (1980), FEBS Letters 118, 274-278, reported the preparation of a covalent conjugate of EGF and diphtheria toxin. In addition, Cawley and Herschman, (1980), Cell22, 563-570, and Simpson, et al., (1982), Cell 29, 469-673, reported on conjugates of EGF covalently linked to the polypeptide A chain of ricin, or to diptheria toxin, by a disulfide bridge. These conjugates containing EGF generally showed high but unpredictably variable toxicity for various normal cells.

[For techniques of preparing or isolating EGF from various species, including the human form (also called urogastrone), or other variants or analogues thereof, and for discussion of their properties, see WO85/00369 (Chiron); WO83/04030 (Applied Molecular Genetics); Nishimura, US 4,528,186; EP-A 46,039 (G.D. Searle); EP-A 128,733 (Genentech); Komoriya, WO85/01284; Wakanuga, EP-A 131,888; and Camble, US 3,917,824.]

More recently, additional similar conjugates of non-immunoglobulin peptides and cytotoxins have been reported. For example, Bacha, et al., (1983), J. Biol. Chem. 258, 1565-1570, describes conjugates of thyrotropin-releasing hormone (TRH) and polypeptides related to diptheria toxin, which were toxic to pituitary tumor cells; and O'Keefe and Draper, (1985), J. Biol. Chem., 260 932-937, report on characterization of a transferrin-diphtheria toxin conjugate, which was toxic to mouse cell cultures. Furthermore, Murphy, et al., (1986), Proc. Nat. Acad. Sci., USA, 83, 8258-8262, describes melanoma-selective cytotoxicity of a genetically fused protein which is equivalent to a covalent conjugate of the apha melanocyte-stimulating hormone (MSH) and a cytotoxin related to diphtheria toxin. JP 60163824 (Nippon Shinyaku KK) discloses a drug carrier, wherein the carrier comprises a protein, such as an apolipoprotein obtained from a serum lipoprotein (e.g., LDL), and a lipid, designed for selectively carrying the pharmaceutical ingredient to the necessary tissue.

Again, the number of toxin molecules that may be directly attached, as taught above, to a molecule of a peptide hormone or growth factor is limited by the need to preserve receptor binding activity. Furthermore, such conjugates of a non-immunoglobulin and a cytotoxin may be excessisvely toxic for normal cells bearing the appropriate receptors.

No admission is made that any of the foregoing references constitute "prior art" and all descriptions are based on the publications rather than on firsthand knowledge of the work described.

SUMMARY OF THE INVENTION

The invention resides in destroying tumor cells with a novel drug conjugate comprising:

(a) A first moiety, "a homing agent", a non-immunoglobulin which preferentially binds to a tumor cell receptor, and is internalized by the cell, and

(b) A second moiety, covalently linked to the first moiety, and comprising a biodegradable polymeric carrier to which one or more cytotoxic molecules are attached. The degradation of the carrier by intracellular enzymes releases a cytotoxic agent, resulting in destruction of the cell. Preferably, the first moiety, besides specifically binding to the tumor cell, also promotes the internalization of the conjugate by that cell. It is also desirable that the carrier be particularly susceptible to tumor cell-associated intracellular enzymes in order to increase the therapeutic index of the conjugate.

The homing agent is preferably a peptide or protein growth factor. One particularly preferred class of homing agent includes mouse Epidermal Growth Factor, and other related growth factors, such as human EGF (urogastrone), alpha transforming growth factor (TGF alpha), and vaccinia virus growth factor, which bind preferentially to tumor cells bearing EGF-binding receptors and are efficiently internalized by those receptors. The growth factor may be purified from natural sources, sythesized by stepwise peptid

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synthesis, or isolated from cells genetically engineered to express th factor. Th agent may be a naturally occurring factor, a synthetic duplicate of that factor, or an active analogu or fragment of the factor. It may also be a substance which is a "pro" form of the factor, which the subject's body converts into the active form of the factor.

It should be noted that the spectrum of tumor cells to which these homing agents bind will differ from agent to agent. Human EGF (urogastron), TGF alpha, and vaccinia virus growth factor, all bind to the same receptor (herein called the EGF receptor), with the same affinity, as does mouse EGF [DeLarco and Todaro, (1978), Proc. Natl. Acad. Sci USA 75 , 4001-4005], and therefore are expected to exhibit the same tumor specific binding as mouse EGF. Furthermore, as the names imply, EGF and related growth factors generally stimulate replication of cells when they bind to the EGF receptor. In addition, TGF alpha is produced by many solid human tumors [Derynck et al., (1987), Cancer Res. 47, 707-712)] in which it is thought to act as a self-stimulating (autocrine) growth factor [Sporn and Todaro, (1980), N. Engl. J. Med. 303, 878-880]. Delivery of a cytotoxic agent linked to TGF alpha (or any other EGF-related peptide) should be selectively toxic to these tumors, not only because of the receptor-mediated uptake of the agent, but also because the mitogenic action of the growth factor should induce increased susceptibility to antimitotic drugs. In other words, the homing agent can render cells which carry receptors more sensitive to drug killing action after it has entered the cells.

In particular, several investigators have demonstrated the presence of EGF receptors in high concentrations on many squamous cell carcinomas, as well as on other carcinomas, and also on sarcomas. Such results include breast, lung, brain and skin tumors. [See the following references: Liberman et al., (1984), Cancer Res 44, 753-760; Merlino et al., (1984), Science 224, 417-419; Kamata el al., (1986), Cancer Res. 46, 1648-1653; Singletary et al., (1987), Cancer Res 47, 403-406; Hunts et al., (1985), Jpn. J. Cancer Res. 76, 663-660; Barknecht et al., (1985), Dermatologica 171, 16-20.]

Other peptide factors, which are unrelated to EGF, may also serve in this invention as homing agents for different tumor cells. For instance, Platelet-Derived Growth Factor (PDGF) is a dimer composed of two related peptides, each with a MW of about 30,000 D, which is released from platelet granules when blood clots form. It is required by smooth muscle cells, fibroblasts and other mesenchymal cells, but not by epithelioid or hematopoetic cells (Cochran and Stiles, (1983), Cell 33, 939-347). After binding to receptors on a cell, PDGF is internalized [Nilsson et al., (1983), Proc. Natl. Acad. Sci 80 , 5592-5596]. A fusion protein containing 80 amino acids of the SV40 t antigen fused to the amino terminus of one chain of PDGF was active in internalization [Wang and Williams, (1984), J. Bio. Chem. 259, 10645-10648], indicating that PDGF, like EGF, could be expected to carry material preferentially into cells bearing appropriate receptors.

PDGF is alos known to be produced by some tumor cells in culture, including glioma, éosteosarcoma, embyronal carcinoma, and a variety of neoplastically transformed fibroblasts [Van Zoilen et al., (1985), Mol. Cell. Biol. 5 , 2289-2297], which suggests a possible self-stimulatory role like that postulated for TGF alpha. Thus PDGF may be useful for homing and internalization of cytotoxic conjugates of the invention into these tumors.

Another possible homing agent, Nerve Growth Factor (NGF), which is a peptide of about the same MW as EGF, has little or no effect on differentiated glial cells, but significantly retards growth of undifferentiated glioma cells [Vinores and Koestner, (1980), Cancer Lett 10, 309-318]. NGF has been observed to decrease the specific induction of neural tumors by ethylnitrosourea [Vinores and Perez-Polo, (1980), J. Cancer Res. Clin. Oncol., 98, 59-63] and to reverse morphologic transformation of anaplastic gliomas [Koestner, (1985), Toxical. Pathol. 13, 90-104]. Thus it appears that some brain tumors have the ability to recognize and respond to NGF.

Because normal differentiated glial cells are refractory to NGF, cytotoxic agents conjugated to NGF would be expected to preferentially destroy brain tumor cells which respond to NGF. Antibody to NGF has been used to diagnose and treat melanoma (Ross et al., EP 202,005; US 723,760), which suggests that these tumors may also respond to NGF.

Transferrin could also be used as a homing agent to deliver cytotoxic drugs to tumors because, like EGF, transferrin-receptor complexes are internalized [Karin and Mintz (1981) J. Boll. Chem 256: 324-3252]. O'Keefe and Draper, (1985), J. Biol. Chem., 260, 932-937, reported that toxicity of a transferrin-diphtheria toxin conjugate was abolished in the presence of excess transferrin, which indicates that this conjugate was internalized via transferrin receptors. As disclosed by Pastan (US 4,545,984), antibodies to the transferrin receptor can be used to deliver toxins to tumors, particularly adult T cell leukemia. Transferrin is also used in radioimaging of tumors [NTIS Tech Note, (1985), NTN85-0891], which indicates preferential binding of transferrin to th s tumors.

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Additional peptide factors may also serve as the homing agent in the invention. For example, alpha melanocyte-stimulating hormone (alpha MSH), another small peptid hormone, was gen tically fused to a cytotoxic protein, and the fusion protein reportedly was cytotoxic for melanoma cells [Murphy, et al., (1986), Proc. Nat. Acad. Sci., USA, 83, 8258-8262]. Also, thyrotropin-releasing hormone (TRH), a tripeptide, has been conjugated with polypeptides related to diphtheria toxin, and the resulting conjugates appeared to be internalized only by cells bearing TRH receptors [Bacha, et al., (1983), J. Biol. Chem. 258. 1565-1570].

In selecting a homing agent, the following desiderata should be considered:

a) the homing substance must be highly specific for tumor cells, as opposed to normal cells;

b) the homing substance should bind to a receptor found in a wide range of tumor cells;

c) the homing substance should promote the internalization (receptor interaction) of the conjugates;

d) the homing substance should stimulate cell proliferation and/or metabolism, therefore rendering the gargeted cell more vulnerable to antimitotic or other antimetabolic action of the cytotoxin;

e) the homing substance should be sufficiently small to penetrate solid tumors efficiently;

f) suitable chemistry must exist for coupling the cytotoxic compound carrier to the homing agent.

The second moiety in the conjugate of the invention is a polymeric carrier for the cytotoxic agent and comprises a plurality of repeating units, each unit having the structure shown in formula (I) below:

$$--CO--CH--NH-- \atop \underset{\underset{CO--A_x--T}{|}}{(CH_2)_p} \qquad (I)$$

wherein p is preferably 1 or 2, where x is preferably in the range of 0-20, where A is a linker, preferably an amino acid, and where, if x is greater than 1, the amino acids A_x may be the same or different. T is either hydroxyl or a cytotoxic substance. The units may be identical, or they may vary in p, x, A_x, or T. Of course, for the conjugate to have a cytotoxic effect, at least one of the units must bear a cytotoxic molecule.

The conjugate per se is preferably of reduced cytotoxicity to normal cells as compared to the substance T in its free form. Intracellular release of T in cytotoxic form is accomplished by cellular enzymes, preferably enzymes preferentially expressed in tumor celles.

The introduction of the side-chain extension A_x, where A may vary among the common amino-acids, and x is preferably in the range 0-20, involves the usual techniques of peptide synthesis. It is assumed that the preferential manifestation in malignant cells of the cytotoxicity typical of the free cytotoxic substance is a function of intracellular enzymatic degradation of the conjugate, liberating either the cytotoxic substance or active derivatives thereof. Preferential release within malignant cells may thus be governed by a differential in terms of type or enhanced level of enzymatic activity between malignant and normal cells.

An elevated level of proteolytic activity, including γ-glutamyl transferase acitivity is associated with a variety of tumors [see W.A.R. van Heeswijk, et al., (1984), in Recent Advances in Drug Delivery Systems, Proc. Int. Symp., 1983, Plenum Press, N.Y., Anderson, J.M. and Kim, S.W., eds., pp 77-100], possibly allowing for tumor specific release of the cytotoxic drug from secondary amide linkage directly with the γ-carboxyl of either the glutamic acid side chain of polyglutamic acid or from such linkage with a glutamyl residue within, or terminal in, A_x. In addition, such enzymatic cleavage at the polyglutamic acid γ-carboxyl, or general proteolytic cleavage within A_x or of the polyglutamic acid polymer backbone, may release a peptidyl prodrug of the cytotoxic substance subject to further processing by lysosomal enzymes, and involving the γ-glutamyl transferase when the drug is γ-linked to glutamic acid in A_x.

The extent and rate of endocellular drug release may thus be significantly affected by the variation in the length and nature of the A_x moiety. Such processing by lysosomal enzymes of bovine serum albumin-daunomycin conjugate has been shown by A. Trouet, et al. [(1982), Proc. Natl. Acad Sci., 79, 626; and (1980), J. Med. Chem., 23 , 1166-1171] to vary widely with the length and sequence of the peptide moiety interspersed between the protein and the drug.

The cytotoxic substance is selected from agents inhibitory of DNA synthesis and function (e.g., adriamycin, daunomycin, bleomycin, melphalan, chlorambucil, cisplatin), of microtubule (mitotic spindle) formation and function (e.g., vinblastine, vincristine), or antimetabolites (e.g., methotrexate), or cytotoxic substances of these or other mechanisms of action For compilations of antineoplastic drugs, see: A
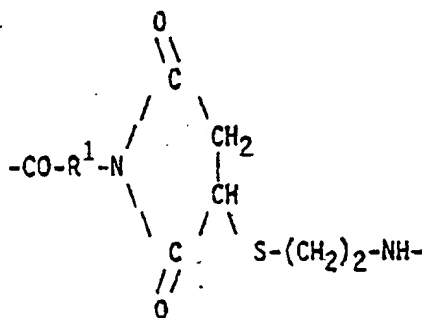
Synopsis of Cancer chemotherapy, R. T. Silver, R. D. Laup r, and C. I. Jarowski, Dunn-Donnalley Publishing Corp., N.Y., 1977; Cancer Treatm nt Symposia: Compilation of Phase II Results with Singl Antineoplastic Agents; NIH Publication No. 85-2739, 1985; NCI Investigational Drugs: Pharmaceutical Data, NIH Publication No. 86-2141, 1986.

5   Preferably at least 10 percent of the units of formula (I) bear a cytotoxic agent (not necessarily th same one for all units). However, higher levels of substitution are pr ferred unless they significantly reduce the cytospecificity of the conjugate.
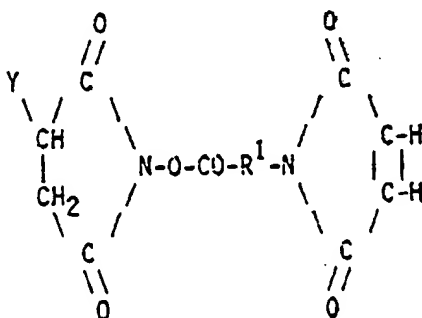
The number of repeating units is not fixed, but is preferably in the range or 20-300. The MW of the polymeric carrier is preferably high enough so that, even if it becomes detached from the homing 10 substance before entering tumor cells, it does not freely enter cells lacking receptors for the homing agent, by passive mechanisms; but rather it should only enter cells actively, by receptor-mediated endocytosis. The polymeric character of the drug construct should prevent its penetration of heart tissue, mitigating against a major shortcoming of daunomycin and other antitumor drugs.

On the other hand, the preferred molecular weight (MW) of the total conjugate is in the range of 10,000 15 to 100,000 D, and less than 50,000 is especially preferred. The MW of the conjugate must be low enough so that it may penetrate solid tumors efficiently. It is known that Fab fragments of antibodies penetrate tumors better than the intact antibodies. [See Delabye, et al., (1986), J. Clin. Invest. 77: 301-11; Buchegger, et al. (1983), J. Exp. Med 158: 413-27; Buchegger et al. (1986), Cancer,58, 655-61.] IgG antibodies have MWs of about 150,000 D prior to addition of any carrier molecules, and even Fab fragments, which are 20 somewhat smaller, may be still be unable to penetrate tumors efficiently after conjugation with a carrier. By comparison, the prepared conjugates of the invention typically had a total MW of about 46,000 D, where the EGF is about 6,000 D and the polymer contributes most of the remaining 40,000 D. Free daunomycin has a MW of about 500 D, so several moles could be attached to each conjugate while maintaining the preferred MW range.

25   The aforementioned homing agent and polymeric moieties are conjugated either directly by peptide linkage, a secondary amide linkage or amino-acids, as in peptides and proteins, or, preferably, by a bridging unit of formula:

$$
\begin{array}{c}
O \\
\backslash\backslash \\
C \\
/ \quad \backslash \\
-CO-R^1-N \qquad CH_2 \\
\backslash \qquad | \\
\backslash \qquad CH \\
\backslash \quad / \backslash \\
C \quad S-(CH_2)_2-NH- \\
// \\
O
\end{array}
$$

where R¹ derives from a heterobifunctional reagent of formula:

$$
\begin{array}{c}
O \qquad\qquad O \\
// \qquad\qquad \backslash\backslash \\
Y \quad C \qquad\qquad C \\
\backslash / \quad\backslash \qquad / \quad\backslash \\
CH \qquad \backslash \qquad / \qquad C-H \\
| \qquad N-O-CO-R^1-N \qquad | \quad | \\
CH_2 \qquad / \qquad\qquad \backslash \qquad C-H \\
\backslash \quad / \qquad\qquad \backslash \quad / \\
C \qquad\qquad\qquad C \\
\backslash\backslash \qquad\qquad\qquad // \\
O \qquad\qquad\qquad O
\end{array}
$$

where R1 is normally, but not restricted to: alkylene (C₁-C₄), preferably (C₃); phenylene, preferably (1,3-); cycloalkylene-alkylene, preferably cyclohexylene-4-methylene; or alkylene phenylene, preferably alkylene

(C₁-C₄)-phenylene (1,4); and where Y = H or SO₂ONa. Other heterobifunctional reag nts differentiating reactive functions of the homing agent and polym ric moiety may als be employed.

In the most preferred embodiment, the therapeutic conjugate of the present invention is a conjugate of EGF with a polyglutamic acid carrier bearing one or more mol cules of daunomycin. Such a conjugate
5 should offer three fold specificity:

(a) specificity for tumor cells having high concentrations of EGF receptors, since these will more effectively bind and internalize the conjugate

(b) specificity for tumor cells having high levels of the enzyme γ-glutamyl transpeptidase since these will more readily degrade the PGA and thereby liberate the daunomycin; and
10 (c) specificity for rapidly dividing tumor cells, since daunomycin is an antimitotic agent.

While it is of course desirable that the conjugate bind to all tumor cells and to no normal cells, it will be recognized that conjugates which bind only to certain tumor cells, and which bind to certain normal cells, may still show sufficient specificity for tumor cells to be of therapeutic activity.

Though in its preferred embodiment, the targeting agent of the conjugate also promotes internalization,
15 it is conceivable that these functions could be performed by different moieties.

The text of the appended claims is hereby incorporated by reference into this specification as an enumeration of the preferred embodiments.

20 ## BRIEF DESCRIPTION OF THE FIGURES

Figure 1a is a photomicrograph of a culture of A431 human squamous carcinoma cell line used to test the effect of the compounds of the invention. The presence of large amounts of EGF receptors thereon is indicated by indirect immunoperoxidase staining (dark color).
25 Figure 1b is a photomicrograph similar to that of Figure 1a, but of a culture of normal W138 embryonic fibroblast cells used as a control. The low level of EGF receptors is indicated by the near absence of staining (light color).

Figure 2a is a photomicrograph of an A431 cell line culture after a 48-hour incubation of 1 μg/ml of free daunomycin and testing for dead cells with Trypan Blue exclusion dye.
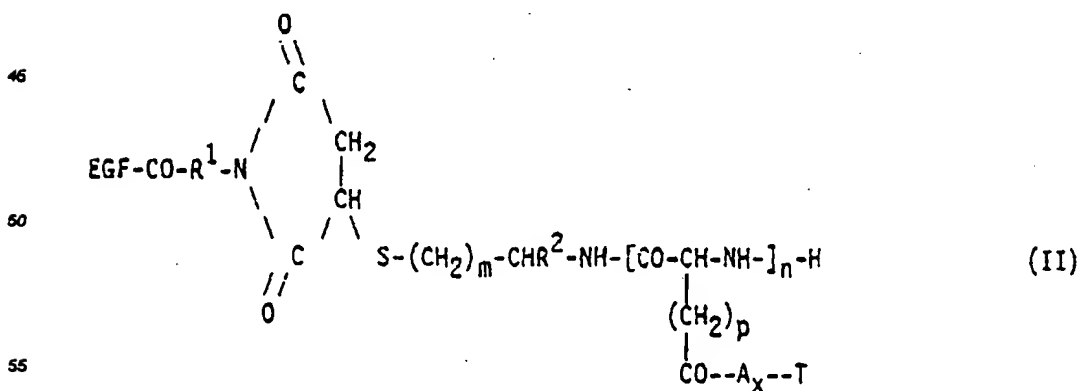30 Figure 2b is a photomicrograph similar to that of Figure 2a, but after 48-hour incubation with, instead, 1μg/ml of daunomycin in the form of conjugate VIII. The dark areas represent the killed cells.

Figure 3a is a photomicrograph at time zero of a mixed culture of A431 and W138 cell lines. The round cells are the tumor cells.

Figure 3b is a photomicrograph similar to that of Figure 3a showing the situation after 48-hour
35 incubation with 1 μg/ml of conjugate VIII. The round tumor cells have greatly diminished.

DETAILED DESCRIPTION OF THE INVENTION

40 The preferred new therapeutic compounds of the invention, using EGF or analogs as homing vector are of formula II:

$$
\begin{array}{c}
O \\
\backslash\backslash \\
C \\
/ \quad \backslash \\
EGF\text{-}CO\text{-}R^1\text{-}N \quad \quad CH_2 \\
\backslash \quad \quad CH \\
\backslash \quad / \ \backslash \\
C \quad \quad S\text{-}(CH_2)_m\text{-}CHR^2\text{-}NH\text{-}[CO\text{-}CH\text{-}NH\text{-}]_n\text{-}H \\
// \quad \quad \quad \quad \quad \quad \quad \quad \quad \quad | \\
O \quad \quad \quad \quad \quad \quad \quad \quad \quad (CH_2)_p \\
\quad \quad \quad \quad \quad \quad \quad \quad \quad \quad | \\
\quad \quad \quad \quad \quad \quad \quad \quad \quad CO\text{--}A_x\text{--}T
\end{array} \quad\quad (II)
$$

where m is 1 or 2, n is 20-300, p is 1 or 2, x is in the range 0-20, R₁ is alkylene (C₁—C₄), 1,3-phenyl ne,

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cyclohexylene-4-methylene, or alkylene (C_1-C_4)-phenylene (1,4); and R_2 is H, or carboxyl, A is an amino-acid residue, or other bivalent moiety introduced to provide functionality for coupling of a cytotoxic substance, and T is hydroxyl or a cytotoxic substance. The monomer units of the polymeric moiety of formual III:
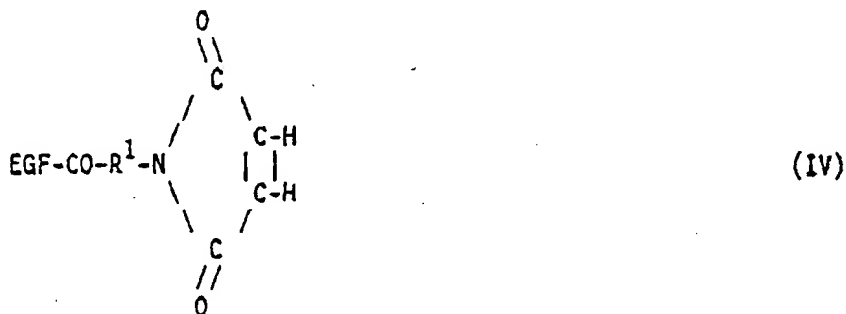
$$-[CO-\underset{\underset{\underset{CO-A_x-T}{|}}{(CH_2)_p}}{CH}-NH-]_n-H \qquad (III)$$

may vary in p, x, A_x, or T, with T being a cytotoxic substance in at least one of the units.

EGF, a single polypeptide of 53 amino acids, can be extracted from submaxillary glands of mice [see J. Savage et al., (1972) J. Biol. Chem. 247, 7609] or it can be synthesized chemically, or by genetic engineering methods, for example using a cloned gene. EGF can also be of human origin.

The cytotoxic substance T and A_x moiety of formula II are normally covalently linked, the nature of the linkage dependent upon the functionality employed in the T and A_x components. Primary amine functionality in the cytotoxic substance T (adriamycin, daumomycin, bleomycin, and melphalan) is normally employed in secondary amide linkage with a terminal carboxyl of A_x. Cytotoxic substances bearing the hydroxyl function (as in vinblastine and vincristine) are linked similarly as esters, or through carbonate linkage (A = an amino-alcohol residue, x = 1), as exemplified by the linkage of norethindrone to poly (N-3-hydroxypropyl-glutamine [see R.V. Petersen et al., (1979) Polymer Preprints, 20, 20]. Carboxylic acid functionality in the cytotoxic substance (as in methotrexate and chlorambucil) is brought into secondary amide linkage with side-chain amine functionality in A_x (e.g., A_x includes a diamino-acid residue), or into ester linkage (A = an aminoalcohol residue, x = 1).

The cytotoxic substance T may also be a cytotoxic platinum complex analogous to cisplatin. The co-ordinate linkage of platinum compounds by the polymeric carrier may be achieved by the utilization as ligand of the γ-carboxy moiety of polyglutamic acid, or of amine or amino-acid or other ligand moieties incorporated in A_x; see Amon, EP-A 190,464.

In formual II-R^1 is a group deriving from a heterobifunctional reagent employed in the elaboration of the linkage conjugating the preferred homing agent EGF and the polymeric moiety III. The conjugation is effected by the coupling of an N-substituted EGF derivative IV:

$$EGF-CO-R^1-N\underset{\diagdown}{\overset{\diagup}{}}\underset{\diagup}{\overset{\diagdown}{}}\begin{array}{c}O\\\|\\C\\\\C-H\\|\ |\\C-H\\\\C\\\|\|\\O\end{array} \qquad (IV)$$
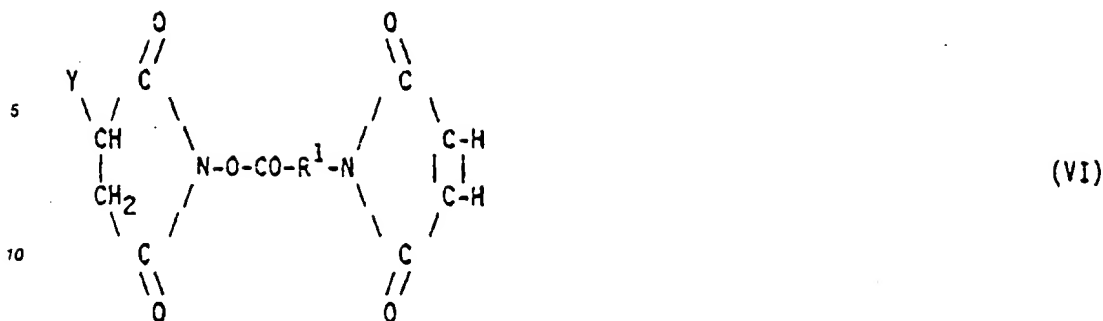
and a derivative (V) of the polymeric moiety III:
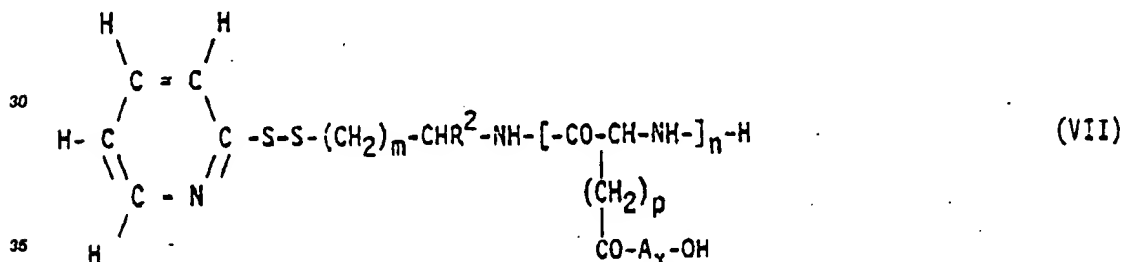$HS-(CH_2)_m-CHR^2-NH-Moiety$ III (V)
where m and R^2 are as defined previously.

The thiol-reactive compound IV is prepared by reaction of EGF with a heterobifunctional reagent VI:

$$Y \begin{array}{c} O \\ \backslash / C \\ CH \backslash \\ | N-O-CO-R^1-N \\ CH_2 / \\ \backslash / \\ C \\ \backslash\backslash \\ O \end{array} \qquad \begin{array}{c} O \\ \backslash\backslash \\ / C \backslash \\ C-H \\ | | \\ C-H \\ \backslash / \\ C \\ // \\ O \end{array} \qquad (VI)$$
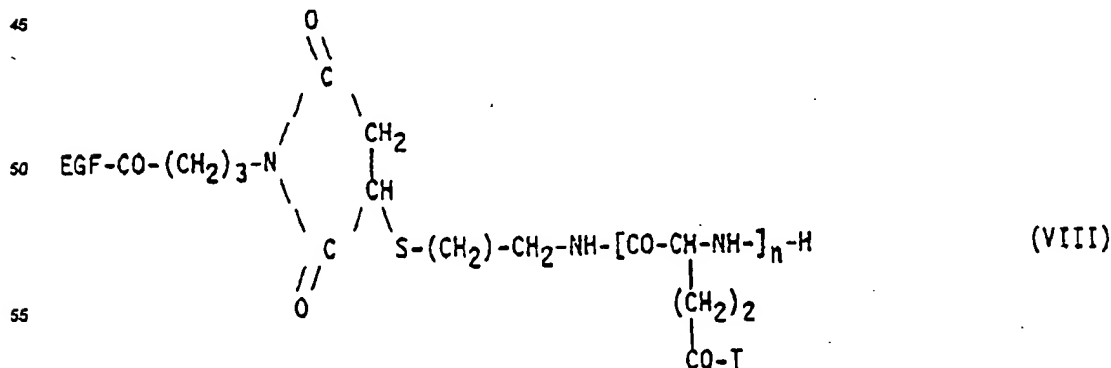
where R^1 and Y are as previously defined. The utility of this, and other reagents (below), resides in the presence of both an acylating moiety, the N-hydroxysuccinimido ester moiety, and an alkylating moiety, the maleimido residue. Other reagents, conforming to this heterobifunctional character, which may be used for the generation of alkylating derivatives of EGF, are m-maleimidobenzoyl-N-hydroxysuccinimide ester, m-maleimidobenzoylsulfo-succinimide ester, succinimidyl-4-(n-maleimidomethyl) cyclohexane-1-carboxylate, succinimidyl1-4-(p-maleimidophenyl) butyrate, sulfosuccinimidyl-4-(N-maleimidomethyl) cyclohexane-1-carboxylate, sulfosuccinimidyl-4-(p-maleimidophenyl) butyrate. A good description of the use of these bridging agents can be found in the Pierce 1985-1986 Handbook & General catalog, page 326 onward. Other heterobifunctional reagents of the above character, or differentiating other functionalities in the components of the conjugate, could also be used.

The thiol-substituted polymer V is prepared following Kato et al., (1984), J. Med. Chem., 27, 1602-7. In brief, this involves the generation of a thiol-masked polymer derivative VII:
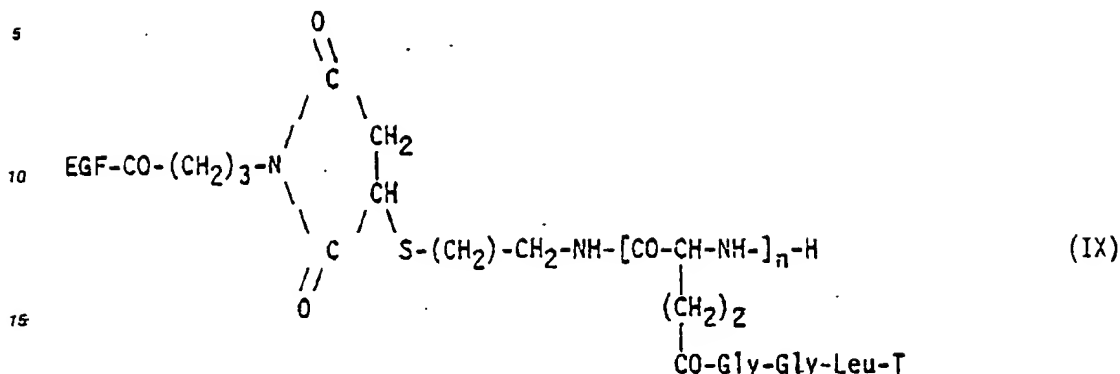
$$\begin{array}{c} H H \\ \backslash / \\ C = C \\ / \backslash \\ H- C C -S-S-(CH_2)_m-CHR^2-NH-[-CO-CH-NH-]_n-H \\ \backslash\backslash // | \\ C - N (CH_2)_p \\ / | \\ H CO-A_x-OH \end{array} \qquad (VII)$$

where m, n, p, x, and R^2 are as previously defined, and partial substitution of the polymer side chains with the cytotoxic substance T, followed by removal of the 2-pyridylthio masking group.

In the preferred case, when T = daunomycin, the incorporation of the cytotoxic substance is achieved by a carbodiimide mediated acylation reaction. Two specific conjugates, which were prepared experimentally, can be illustrated by the following formulas:

$$\begin{array}{c} O \\ \backslash\backslash \\ C \\ / \backslash \\ / CH_2 \\ EGF-CO-(CH_2)_3-N | \\ \backslash CH \\ \backslash / \backslash \\ C S-(CH_2)-CH_2-NH-[CO-CH-NH-]_n-H \\ // | \\ O (CH_2)_2 \\ | \\ CO-T \end{array} \qquad (VIII)$$

where m is 1, n is 20-300, p is 2, x is 0, R_1 is $(CH_2)_3$, R^2 is H, and T is daunomycin or hydroxyl in a ratio of about 1 to 6;

$$
EGF-CO-(CH_2)_3-N
\begin{array}{c}
\quad\quad\quad\quad O \\
\quad\quad\quad\quad \backslash\backslash \\
\quad\quad\quad\quad C \\
\quad\quad / \quad\quad \backslash \\
/ \quad\quad\quad\quad CH_2 \\
\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad CH \\
\backslash \quad\quad / \quad \backslash \\
\quad C \quad\quad S-(CH_2)-CH_2-NH-[CO-CH-NH-]_n-H \\
\quad // \quad\quad\quad\quad\quad\quad\quad\quad\quad\quad | \\
\quad O \quad\quad\quad\quad\quad\quad\quad\quad\quad (CH_2)_2 \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad | \\
\quad\quad\quad\quad\quad\quad\quad\quad\quad\quad CO-Gly-Gly-Leu-T
\end{array}
\quad\quad (IX)
$$

where m is 1, n is 20-300, p is 2, x is 3, A_x is Gly-Gly-Leu, R_1 is $(CH_2)_3$, R^2 is H, and T is daunomycin or hydroxyl in a ratio of about 1 to 6.

The introduction of the side-chain extension A_x, where A may vary among the common amino-acids, and x is preferably in the range 0-20, involves the usual techniques of peptide synthesis. [See, for examples, W.A.R. van Heeswijk, et al., (1985), J. Controlled Release 1, 301-315, and Hoes et al., (1985), J. Controlled Release 2, 205-213.]

Compound II demonstrates very useful properties in cancer therapy. In particular, Compound II is internalized by malignant cells and the cytotoxicity of the cytotoxic substance daunomycin is expressed therein. Its selectivity of action upon malignant cells is greater than that of known cytotoxic drugs. Thus, the first valuable property relates to its strong affinity toward malignant cells over normal cells.

For demonstrating this characteristic, a human squamous carcinoma cell line was selected, for instance A431 cell line shown in Figure 1a [see M.D. Waterfield (1982), J. Cell. Biochem., 20, 149-161], although other tumor cells can be used as well. The membrane of these cells contains a very large concentration of EGF receptors which makes them highly suitable for tests with Compound II. The presence of these receptors is shown by indirect immunoperoxidase staining and appears as dark color areas of Figure 1a. Control cell lines, for instance W138 embryonic fibroblast cells with a low amount of EGF receptors (see Figure 1b), were treated identically for comparison and appear as lighter color areas on the photomicrograph. It should be noted that the suitability of any given tumor for treatment with Compound II where EGF is the homing agent can be determined by means of measuring EGF receptors by tests such as the one described, for instance, in Example 2, below. Receptors for other homing moieties may also be detected by binding of appropriate antibodies or the homing substance itself.

When a known amount of Compound II, in particular Conjugage VII (p = 2; x = 0, T = daunomycin or OH in a ratio of 1 to 6) radio-labelled with I^{125} (iodine is part of the EGF moiety) was incubated with A431 cells using W138 cells as control, 31 percent of the total radioactive iodine was retained in the intra-cellular compartment and 2 percent in the membrane of the tumor cells, while in the control cells, the corresponding values were 2 percent and 0 percent, respectively. Using free EGF in place of Conjugate VII in these experiments gave the corresponding 14 percent/0 percent and 7 percent/0 percent values instead. The reason for the greater accumulation of Conjugate VIII as compared to free EGF is possibly due to retention of EGF by the polymer backbone of Conjugate VIII by endocytic vesicles, whereas EGF is recycled freely back to the medium. Application of tests for internalization of a conjugate by appropriate receptor-bearing cells, such as that described in example 3, below can be used to determine whether or not a the homing substance in a given conjugate has retained its capacity of internalization, and, therefore, may be suitable for treatment of tumors.

A second valuable and unexpected property of Compound II relates to its enhanced toxicity as compared with free daunomycin toward malignant cells. This is illustrated by Figures 2a and 2b.

In Figure 2a, a culture of A431 cells is shown after 48-hrs incubation with a medium containing 1μg/ml of free daunomycin. Viability was scored by means of the Trypan Blue exclusion dy method. Exclusion of the dye from th cells demonstrates the viability th reof. In contrast, when an equivalent amount of daunomycin in the form of Conjugate VIII was used in a similar experiment (see Figure 2b), a very large number of cells were killed as shown by the shrivelled up cells and the dark areas where the dye has accumulated.

The selectivity of Conjugate VIII toward tumor cells was further tested. For instance, figure 3a and 3b show the effect of Conjugate VIII in the case of a mixed culture of A431 and WI38 cells. The round cells represent the A431 tumor cells and the elongated cells are the WI38 control cells. The culture medium was photographed at time zero (Figure 3a). Figure 3b illustrates this situation after 24 hrs in a medium containing 1 μg/ml of Conjugate VIII. It can be seen that the round darker malignant cells have strongly regressed. Testing similar to that described in examples 4, 5 and 7, below, can be performed to determine the enhancement of selectivity and/or potency of any given cytotoxin for any selected tumor cells provided by any desired form of Compound I.

In conclusion, it has been demonstrated that at equivalent molar concentrations (of daunomycin), Conjugate VIII was much more lethal to squamous carcinoma cells that free daunomycin itself. Also, Conjugate VIII has a selective mortal activity on tumor cells, but leaves normal cells alive, whereas under the same conditions, free daunomycin will kill normal cells.

The following examples illustrate the invention in more detail.

Example 1. Synthesis of a Conjugate of EGF and Daunomycin-Grafted Polyglutamic Acid (Conjugate VIII)

Conjugate VIII contained a polymeric compound (VII) with a polyglutamate backbone structure (p = 2, x = 0), in which some γ-carboxylic groups are condensed with daunomycin and having a 2-pyridyldithio-ethylamido heading group (m = 1, R^2 = H), which was prepared according to Y. Kato, et al., 1984, J. Med. Chem., 27, 1602-1607 (compound 5 in scheme II of Kato).

The identity of the compound was checked analytically: MW = 29,000 D by quantitatively determining the 2-pyridyldithio group; ratio of daunomycin to carboxylate = 1/6 as determined by spectrometric quantitation of daunomycin at 480 nm.

To 25 mg of the polymer dissolved in 2 ml of 10mM sodium phosphate buffer at pH 7 were added 0.1 ml of a 0.3 M solution of dithiothreitol (DTT). After 1 hr at 40° C, the solution was dialyzed overnight gainst a 0.1 M sodium phosphate buffer at pH 6.0 (SPECTRAPOR membrane, MW cutoff 3500); this regenerated the thioethylamido group of the molecule (compound 6 in scheme II of Kato); yield 23 mg of a red compound after freeze-drying (poly-(DM)-Glu-SH. The poly-(DM)-Glu-SH polymer was reacted with an excess of thiopropyl-SEPHAROSE in the pyridylsulfide form (4° C; 12 hrs; phosphate buffer, pH 6) and the gel was rinsed with an excess of the same buffer in order to eliminate the polymer lacking the SH extremity. The polymer was conserved in this form in the cold. It was then regenerated by treating the gel with an excess of mercaptoethanol (12 hrs), dialyzed against water (overnight, 4° C), and freeze-dried before reacting with EGF.

One hundred μg of EGF (SIGMA) was dissolved in 500 μl or 10 mM phosphate buffer, pH 7.0, containing 0.14 M NaCl. Then a quantity of 125I-EGF sufficient to provide an activity of 10,000 cpm/μg EGF was added followed by 50 μl of a 32 mM solution of N-succinimidyl-4-(N-maleimido)-butyrate (SMBU) (origin: Sigma), in dimethylformamide. The mixture was allowed to stand for 1 hr at 25° C, then it was dialyzed against a 0.1 M sodium phosphate buffer-0.1 M NaCl, pH 6.0, to eliminate the excess of SMBU.

The desired product (IV, R^1 = $(CH^2)_3$) resulting from the condensation of SMBU and EGF was not isolated, but to the 500 μl of the dialyzed EGF solution were added 1 mg of poly-(DM)-Glu-SH and the mixture was slowly agitated overnight at 4° C. Five ml of thiopropylsepharose (pyridylsulfide form) were then added and the reaction was continued at 4° C for 12 hrs. The gel was washed successively with 3 portions of 1 ml of sodium phosphate buffer, the eluent was concentrated under reduced pressure and subjected to gel filtration on SEPHADEX G75 (column 40 x 0.8 cm), using 0.1 M ammonium carbonate solution, pH 7.0. The fraction containing the EGF-poly-(DM)-Glu conjugate (VIII), detected by absorption at 480 nm, was collected and freeze dried. Yield: 80 μg of solid.

Example 2. Selection of A431 Test Cell Lin s from Evidence of Receptor Concentration on the C ll Membrane by Indirect Immunoperoxidase Staining

Indirect immunoperoxidase staining on cell lines wer performed on trypsinized cells in 35 mm PVC plates. The surface was pre-treated with phosphate buffered salin (PBS) pH 7.2, the excess was then removed and the PBS washed cells (10^5/well in 50 μl PBS) were added to the plated and centrifuged for 5 minutes at 2000 rpm. 50 μl/well of 0.5 percent glutaraldehyde in cold PBS were then added to the dish and incubated for 15 minutes at room temperature. After two roundS of washes with PBS, the wells were filled with 100 mM glycine in a 0.1 percent BSA solution and allowed to stand for 30 minutes at room temperature to block glutaraldehyde activity. After two PBS washes, indirect immunoperoxidase was done by first denaturing the cells with an ice cold mixture of 99:1 ethanol-acetic acid for 30 minutes at 4° C. During this period, 3 μl of antibody were diluted in 1 ml PBS + 1 percent fetal calf serum (FCS). The wells were then washed twice with PBS and incubated for 5 minutes with a solution of 20 percent FCS in PBS. This was then replaced by 200 μl/well of the antibody solution and incubated for 30 minutes at room temperature. The wells were then washed twice with PBS, once with PBS + 0.1 percent Tween, and once again with PBS. 200 μl/well of a 1:400 dilution of swine anti-mouse peroxydase (POD) conjugated antibody (DAKO) in PBS 1 with percent FCS was then added for 30 minutes at room temperature. The wells were then washed twice with PBS, once with PBS + 0.1 percent Tween and twice with distilled water. The in situ coloring was achieved by incubating the cells at room temperature with a solution of 10 ml 0.01 M phosphate buffer, pH 6.0, 5 μl, 35 percent oxygenated water and 100 μl of 1 percent ortho-dianisidin (MERCK) in methanol.

Example 3. Internalization of Conjugate VIII in A431 Squamous Carcinoma Cells Compared to W138 Fibroblasts

The Conjugate VIII, radiolabeled with ^{125}I on the EGF, was incubated with confluent cell cultures of A431 and W138 cells for 6 hrs at 37° C in solution A (4 parts DMEM and 1 part of 50 mM Tris, 100 mM NaCl and 0.1 percent BSA adjusted to pH 7.4). The cells were then washed four times with ice-cold PBS + 1mM CaCl$_2$ and 1 mM MgCl$_2$. Fifty percent trichloracetic acid was added in a proportion of 1:5 to the pooled solution A and PBS, and the mixture was counted on a gamma counter. The cell membrane was destabilized by treatment on ice with 200 mM acetic acid and 150 nM NaCl (solution B) for 6 minutes. The solution B was then removed and the cells washed twice with solution B. These pooled B solutions were assayed for ^{125}I. This treatment releases the EGF receptors bound to the cell surface. The cells were then completely dissolved in 0.2 N NaOH. The radioactivity found there represented the interalized Conjugate VIII. In control experiments, using free EGF ^{125}I, a proportion of the EGF is recycled to the medium, therefore lowering the intracellular EGF. The results on the internalization of ^{125}I labelled EGF and Conjugate VIII in WI38 and A431 cell cultures are given in the following Table 1n terms of counts per min (background 30 cpm) for successively: EGF not integrated in the cell, EGF bound to the membrane, and EGF in the intra-cellular compartment. The percent of total cpm is given in brackets.

Table 1

Internalization of free EGF and Conjugate VIII

		EGF cpm (%)	Conjugate VIII cpm (%)
WI38	Unbound	530 (93)	1169 (98)
"	Membrane bound	29 (0)	28 (0)
"	Intracellular	67 (7)	52 (2)
A431	Unbound	536 (86)	831 (67)
"	Membrane	31 (0)	57 (2)
"	Intracellular	111 (14)	390 (31)

These results show that conjugate VIII is more efficiently internalized in maligant cells (31 percent) than in normal cells (2 percent).

Example 4. Comparative Cytotoxic Effects of Free and Conjugated Daunomycin on A431 Squamous Carcinoma Cell

All cells were maintained in Dulbecco's Modified Medium (DMEM), 10 percent foetal calf serum (FSC) (GIBCO), 2 percent penicillin-streptomycin (Gibco) and 1 percent fungizone (GIBCO) in 5 percent CO_2. They were plated at 50 to 60 percent confluence 24 hours before the addition of the toxin. 1 μg.ml of daunomycin, or equivalent in Conjugate VIII, was added in DMEM 10 percent FCS and the cell death rate visualized by trypan blue exclusion. Four volumes of 0.2 percent (w/v) Trypan Blue in water was freshly mixed with 1 volume of a saline solution 4.25 percent (w/v) of NaCl in water. One volume of this solution was mixed with 1 volume of PBS on the cell monolayer or to 1 volume of cell suspension in PBS. Observation and scoring took place 48 hours after addition of the toxin (see Figure 2a and 2b). These results show that daunomycin is much more effective against malignant cells when in the form of Conjugate VIII than when in the free state.

Example 5. Effect of Conjugate VIII on Mixed Cultures of Tumor (A431) and Normal (WI38) Cells)

Cells were maintained as described in Example 4. The WI38 fibroblasts were first plated and the A431 squamous carcinoma cells were plated the next day. The mixted culture was left growing for 24 hours and then 1 μg/ml of Conjugate VIII was added to DMEM 10 percent FCS. Nearly all the A431 cells were selectively killed after 24 to 48 hours whereas the WI38 fibroblast were left alive. Hence, the mixed culture became free from the tumor cells demonstrating that conjugate VIII can be used to separate the normal cells from cancer cells. The experiment was repeated but using only 0.1 μg/ml of Conjugate VIII (for the controls, equivalents of daunomycin were used in free form). Observation of the cultures after 15 days showed that the test samples contained no more of A431 cells, which situation was confirmed by continuing culturing for 6 weeks under normal conditions, this resulting in no reformation of tumor cells. In contrast, in the controls all cells, malignant and normal, had died after 15 days.

Example 6. Synthesis of a Conjugate of EGF and Glycyl-Glycyl-Leucyl-Daunomycin Grafted Polyglutamic Acid (Conjugate IX)

Conjugate IX contained a polymeric compound (VII) with a polyglutamat backbone structure ($p = 2$, $x = 0$), in which some γ-carboxylic groups ar condensed with daunomycin and having a 2-pyridyldithio-ethylamido heading group ($m = 1$, $R^2 = H$), which was prepared according to Y. Kato, et al., 1984, J. Med. Chem., 27, 1602-1607 (compound 5 in scheme II of Kato).

The polymer was subjected to the procedure reported by Hesswijk, et al., (1985), J. of Controlled Release, 1 (4), 312, for extending the side chain with -Gly-Gly-Leu-(A$_x$ in formula II), as follows: 40 mg of the polymer and 68 mg of saccharin (0.31 mmole) were dissolved in 1 ml of DMF and the solution was allowed to stand for a few hours (solution A).

On the other hand, 0.32 mmole (40 ul) of N,N,'N'-tetramethylguanidine (TMG) were slowly added to a stirred suspension of 0.32 mmole (80 mg) of H-Gly-Gly-Leu-OH in DMF. Stirring was continued until all solids had dissolved (solution B).

Then, 0.43 mmole (70.3 mg) of N,N'-carbonyl-diimidazole were added to solution A and, after stirring for 30 min, solution B was added. The mixture was further stirred for 3 days at room temperature. The mixture was added into 15 ml of 0.1 M phosphate buffer (pH 7.0) and the resulting solution was dialyzed into water (12 hrs), filtered on a millipore membrane (0.45 μm) and the filtrate was freeze-dried. The polymer (yield 80 percent mg) was analyzed by hydrolyzing an aliquot in 6 N HCl for 12 hrs at 180 C. Determination of the amino acids in the hydrolyzate was carried out by High Performance Liquid Chromatography of the amino acid-phtalaldehyde derivatives (detection by fluorescence). The following ratio of glutamic acid:glycine:leucine was measured: 0.95:2:1.

The pyridine-S group was removed with dithiothreitol according to Kato, et al., and analysis was performed by measuring the absorbance at 343 nm of the liberated pyridine-2-thione. Neglecting the presence of the TMG+ ion, a MW of 25,500 was found, meaning that in the product the degree of polymerization is about 75-80.

The introduction of daunomycin was accomplished as follows: 33.7 mg (74.7 umole) of the side-chain extended polymer were dissolved in 15 ml of 2 percent aqueous NaCl and 20 mg (35.5 μmole) of daunomycin hydrochloride were added. The pH was brought to 5.5 with 0.1 N NaOH after which 28 mg (0.1 mmole) of 1-ethyl-3-[3-(dimethylamino)]-carboiimide hydrochloride (EDC) were added under stirring.

After agitation for 18 hrs, the mixture was diluted with 15 ml of 1 M NaCl and dialyzed in water. The residue was freeze-dried which provided 31.6 mg of polymer. Splitting the disulfide with DTT as before and analyzing spectroscopically the pyridine-thione indicated the presence of about 10 DM per molecule, i.e. a ratio of labelled side-chains to unlabelled side-chains of about 1:7.

Conversion of an aliquot of the above disulfide to the desired thiol was done as follows: 2 mg of polymer were dissolved in 2 ml of 0.1 M phosphate buffer (pH 7.0) and 150 μl of 0.3 M DTT were added. After allowing to stand for 1 hr at 42 degrees, the solution was dialyzed for 24 hrs against freshly degassed phosphate buffer (pH 6.0): a SPECTRAPOR bag was used (MW cut-off = 1000 D).

Simultaneously, EGF was activated by taking 0.2 mg of EGF (SIGMA) and dissolving in 0.75 ml of 10 mM phosphate buffer in 0.14 M NaCl, pH 7.0; then adding 0.5 ml of a 10 mg/ml SMBU solution in DMF. After 2 hrs at 20° C, the mixture was dialyzed at 4° C against a 0.1 M Phosphate buffer containing 0.1 M NaCl at pH 6.0 (membrane MW cut-off = 1000).

The overall volume of both the dialyzed polymer and dialyzed EGF solution were reduced to 1 ml by absorption with CMC powder, then they were mixed together and allowed to stand for 24 hrs in one dialysis bag. The mixture was chromatographed on SEPHADEX G75 (eluent 0.1M NH4CO3) and the fractions absorbing at 480 nm were collected and cleaned from unreacted polymer by treating with thiopropyl-sepharose overnight at 4° C. The gel was washed with 0.1M NH4CO3 and 22.5 ml (O.D. of 0.176) of solution was collected. Yield was about 80 percent of Conjugate IX. The respective weight contributions of DM and EGF in the product are about equal.

The presence and binding efficiency of the EGF factor in Conjugate IX was checked by im-munoprecipitation with EGF antibody and attachment of the immunocomplex to a protein A-Sepharose gel (CL4B, Pharmacia). The procedure was as follows: Protein A-Sepharose gel was rehydrated to provide a 50 percent (V/V) solution in NET-NP40 buffer (100 nM NaCl, 1 mM EDTA, 1 mM EDTA, 10mM Tris, pH 7.5, 0.5 percent NP40). Bovine serum albumin (BSA) was added to 120 μl of the buffered sepharose solution to provide a 0.3 percent (by weight) BSA solution (S). On the other hand, BSA was added to 12 ul of a solution of antiserum against EGF (Collaborative Research) so as to provide a 0.3 percent by weight BSA solution in antiserum (Ab). Both (S) and (Ab) were incubated overnight at 4° C, then a quantity of Conjugate IX corresponding to 120 μg of daunomycin was added to sample Ab and incubated for 7 hrs at 4° C under

agitation. Then solution (S) was added and the mixture was agitated per 12 hrs at 4° C. A control (C) was prepared by adding th same quantity of Conjugate IX to another identical sample of solution (S). Both the above mixture (M) and the control wer centrifuged for 5 min at 2000 rpm and the absorbance (A) of th supernatant liquid measur d at 480 mm. The results are 0.41 for M and 0.955 for C, which shows that Conjugate IX contains EGF, the conformation of which is recognized by the antibody on the gel.

Example 7. Toxicity of Conjugate IX and of Free Daunomycin Toward A431 Malignant and W138 (Control) Cells

The toxicity of conjugate IX and of free daunomycin toward A431 malignant and W138 (control) cells was compared. The effect of these drugs was evaluated by the degree of inhibition of cellular protein synthesis. For this, we measured the level of incorporation of ^{35}S methionine (^{35}S-met NEN) into newly synthesized proteins after a 48 hrs exposure to different concentrations of the drugs.

Cells were plated on 1.5 cm Petri dishes at 50 percent to 60 percent of confluence before the addition of the toxin. 0.1, 0.5 or 1 μg/ml doses of daunomycin (controls) or its equivalents in the form of Conjugate IX were added in DMEM 10 percent FCS. Cell death consecutive to this addition was measured as follows: the cells were exposed for 1 hour at 37° C in 500 μl DMEM low methionine medium (GIBCO) containing radioactive ^{35}S met. The medium was then removed and the dishes were washed with PBS 9137 mM NaCl, 2.7 mM KC1, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.2). These solutions were stored for beta counting. The cells were lysed with 1 ml 0.1N NaOH and placed in a tube with 500 μl Trichloroacetic acid (TCA) 10 percent to precipitate the proteins. This mixture was filtered on GF/A filters (Whatman) to eliminate unreached ^{35}S-met. The filters were washed twice with 1 ml of 10 percent TCA and once with 100 percent ethanol. The filters were dried at 80° C for 2 hours and subjected to beta counting in Econofluor (NEN) scintillation medium. The results reported in the following table 2 Show that Conjugate IX is very cytospecific but less cytotoxic than daunomycin alone.

Table 2

Toxicity of Conjugate IX and of Free Daunomycin

	Drug Concentration (μg/ml)	% of protein Synthesis Inhibition A431	WI38
Free Daunomycin	0.1	30	10
	0.5	68	46
	1.0	97	80
Conjugate IX	0.1	4	0
	0.5	17	6
	1.0	47	4

Example 8. In Vivo T st of Conjugate VIII

We injected 5 x 10⁶ A431 cells nude mice, and when the tumors reach d 3 to 5 mm, w injected into two sets of nude mice 0.1 mg/kg of daunomycin either in th form of conjugate VIII (see example 1) or in free form. In the prior art, up to 10 mg/kg dos s have been used in mice (S. Schwarz, et al., 1975, Cancer Chem. Rep., 6, 2, 107-114). The drug was injected either directly in the tumor or in the caudal vein 4 tim s at 3 days intervals. The measures were taken a week after the last injection.

By visual inspection of the animals, it was noted that the growth of the tumors treated with conjugate VIII was significantly reduced, as compared to control mice or to treatments with free daunomycin. These results were similar whether the injection of the drug was directly in the tumor or in the caudal vein which shows that biodegradation and release of the cytotoxic substance occurs substantially only in the target cells. Results are shown in the following table 3.

Table 3

In Vivo Test of Conjugate VII

	Injection	Initial Surface of the Tumor (A)	Final Surface of the tumor (B)	Growth coefficient (B/A)
Daunomycin	I.V.*	30.5	500	16.4
Conjugate VIII	I.V.	25	54	2.2
Daunomycin	I.T.**	9	170	18.7
Conjugate VIII	I.T.	9	50	5.6
Control***		9	320	35.6

* I.V. = Intra-venous.
** I.T. = Intra-tumor
***Placebo injection

At the end of the experiment, the tumors were dissected and weighed. Results are given below. If corrections for slight differences of tumor size at the beginning of the treatment are taken into account, it can be seen that the results (Table 4) correlate well with the surface estimate given in the previous Table 3.

Table 4

In Vivo Test of Conjugate VII (Tumor Weights)

Daunomycin	I.T.	:	1.178 g
Conjugate VIII	I.T.	:	0.359 g
Daunomycin	I.V.	:	6.789 g
Conjugate VIII	I.V.	:	0.816 g

In conclusion, the compounds of the invention show better performances in selectively killing squamous carcinoma cells than daunomycin, both in vitro and in vivo. In the animal tests, we not d that very low amounts of conjugate VIII hav a remarkable effect on tumor growth.

Example 9. In Vivo Test of Conjugate IX

The in vivo tests reported in Example 8 were repeated using Conjugate IX. Thus, 2 mg/kg of daunomycin, free or in the form of Conjugate IX were injected every 3 days over 9 days in the caudal vein of nude mice bearing A431 tumors. The tumor growth inhibition by conjugate IX was significantly greater than by free daunomycin (D), free EGF or DM labelled but untargeted polymer (DMA). These results are shown in the Table 5 below where the values correspond to tumor diameter (in mm) measured with a calliper after a number of days. The lethal dose of Conjugate VIII and Conjugate IX have not been measured but are presumably less toxic than free daunomycin which enters freely into most normal cells.

Table 5

In Vivo Test of Conjugate VII

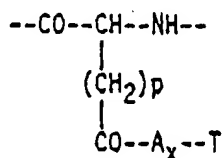| Days:	0	4	7	11	14
Control	10	14	17	22	24
Daunomycin	10	12	15	20	21
Free EGF	10	13	14	17	17
Polyglu-DM	10	11	12	16	17
Conjugate IX	10	11	12	14	15

Claims

1. A therapeutic composition comprising a chemical conjugate including a first moiety, other than an immunoglobulin or fragment thereof, which preferentially binds to a tumor cell, and a second moiety linked to the first moiety, said second moiety comprising a plurality of repeated units, each unit having the structure

$$--CO--CH--NH--$$
$$(CH_2)p$$
$$CO--A_x--T$$

where p and x are integers, where A_x is a bifunctional linker, and where T is selected from the group consisting of free hydroxyl and a cytotoxic substance,
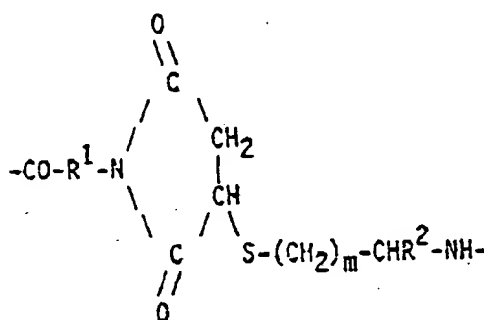
where said units may be the same or may vary, T being a cytotoxic substance in at least one of said units, and where said conjugate is internalized by tumor cells.

2. Th composition of claim 1, where p is 1 or 2, wher x is in th rang of 0-20, wher A_x is an amino acid introduced to provide a functional group for convenient coupling of various cyctotoxic substances, and where if x is greater than 1, th amino acids A_x may be the sam or different.

3. Th composition of claim 1 where th first moiety is selected from the group consisting of mouse epidermal growth factor (EGF), other animal EGF species, human EGF (also known as urogastrone), alpha-transforming growth factor (TGF), vaccinia virus growth factor (VGF), and any other peptides which bind preferentially to tumor cell rec ptors for EGF and ar internalized by those receptors.

4. The composition of claim 1 where the first moiety is selected from the group consisting of nerve growth factor (NGF), platelet-derived growth factor (PDGF), melanocyte-stimulating hormone (MSH), thyrotropin releasing hormone (TRH), transferrin, low density lipoprotein (LDL), and any other non-immunoglobulin substances also bound by tumor cell receptors of the foregoing substances.

5. The composition of claim 1 wherein the first moiety is a substance which promotes internalization of the conjugate by a tumor cell.

6. The composition of claim 1 wherein the first moiety is EGF.

7. The composition of claim 1 where the EGF is essentially identical to human EGF.

8. The composition of claim 1 wherein the first moiety has a mitogenic effect on a tumor cell.

9. The composition of claim 1 wherein the first moiety is directly linked to the second moiety by means of a peptidyl linkage.

10. The composition of claim 2 wherein the first moiety is linked to the second moiety by a structure having the formula:

$$
-CO-R^1-H
\begin{array}{c}
O \\
\backslash\backslash \\
C \\
/ \quad \backslash \\
/ \qquad CH_2 \\
\qquad | \\
\backslash \qquad CH \\
\backslash \quad / \quad \backslash \\
C \qquad S-(CH_2)_m-CHR^2-NH- \\
// \\
O
\end{array}
$$

where R^1 is: alkylene (C_1-C_4); 1,3-phenylene; cyclohexylene-4-methylene; or alkylene (C_1-C_4)-phenylene (1,4); R^2 is H, or carboxyl; and m = 1 or 2.

11. The composition of claim 1, where the molecular weight is also less than about 100,000 D.

12. The composition of claim 1, wherein T is a cytotoxic substance in at least about 10% of the units.

13. The composition of claim 1 wherein the cytotoxic substance is an inhibitor of DNA synthesis and function.

14. The composition of claim 10 wherein the cytotoxic substance is selected from the group consisting of anthracycline drugs, bleomycin, melphalan, chlorambucil, and cis-platinum.

15. The composition of claim 14 wherein the substance is selected from the group consisting of adriamycin, daunomycin, and daunorubicin.

16. The composition of claim 1 wherein the cytotoxic substance is an inhibitor of microtubule formation and function.

17. The composition of claim 16 wherein the cytotoxic substance is selected from the group consisting of vinblastine and vincristine.

18. The composition of claim 1 wherein the cytotoxic substance is an antimetabolite.

19. The composition of claim 18 wherein the cytotoxic substance is methotrexate.

20. The composition of claim 1 wherein the cytotoxic substance is a radioactive substance.

21. The composition of claim 1 wherein the cytotoxic substance is an enzymatic inhibitor of metabolism.

22. The composition of claim 21 wherein the cytotoxic substance is selected from the group consisting of the following intact proteins, subunits or modified forms thereof: Recin (particularly the A chain), diptheria toxin, Pseudomonas exotoxin, and abrin.

23. The composition of claim 1 wherein the cytotoxic substance is selected from the group consisting of aureomycin, gelonin, and hematoporphyrin.

24. The composition of claim 1 wherein the cytotoxic substance is daunomycin.

25. A therapeutic method for preferentially destroying tumor cells, comprising administering a covalent conjugate of a cytotoxic substance and a homing substance which non-immunologically and preferentially binds to tumor cells, said cytotoxic substance and said homing substance being conjugated by a polymeric

carrier having a nontoxic, biodegradable, polyaminoacid backbone, and permitting said conjugat to bind to and be internalized by said tumor cells, wh rein said polyaminoacid backbone is degraded, releasing said cytotoxic substance.

26. The method of claim 25 wherein said polyaminoacid backbone is selected to control the rate of biodegradation and hence the rate of release of the cytotoxic substance.

27. The method of claim 25 wherein said polyaminoacid backbon is pref rentially degraded by tumor cells.

28. The method of claim 25 wherein the conjugate is essentially incapable of penetrating normal cells.

29. The method of claim 25 wherein said homing substance promotes the internalization of the conjugate by the tumor cells.

30. The method of claim 25 wherein the conjugate is of reduced cytotoxicity to normal cells relative to the free cytotoxic substance.

31. The method of claim 25 wherein the tumor cells are carcinoma cells.

32. The method of claim 31 wherein the tumor cells are squamous carcinoma cells.

33. The method of claim 25 wherein intracellular release of the cytotoxic substance in toxic form occurs essentially only in tumor cells.

34. The method of claim 25 wherein the conjugate also increases tumor cell metabolism, thereby increasing its sensitivity to the cytotoxic substance.

35. The method of claim 25 wherein the homing substance is epidermal growth factor.
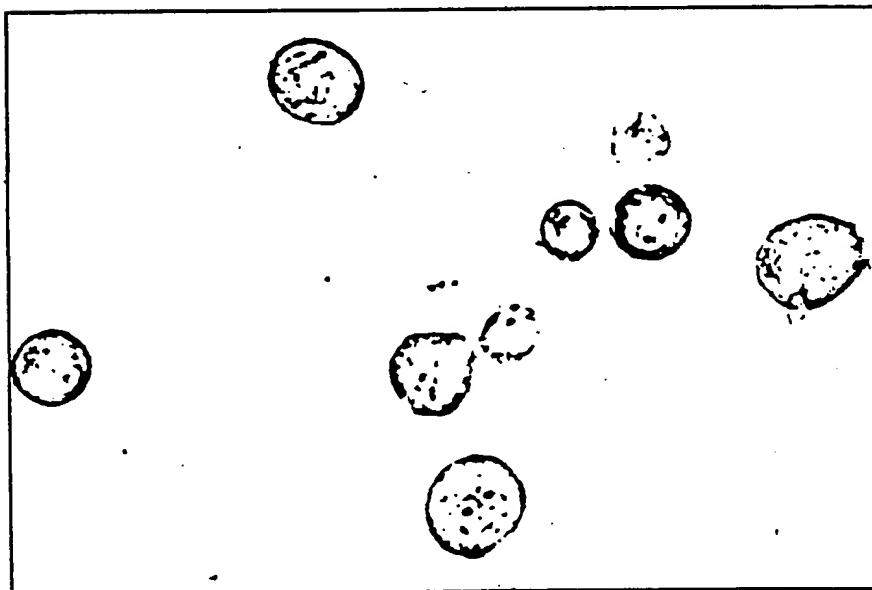
36. A therapeutic composition consrising a chemical conjugate including a first moiety which preferentially binds to a tumor cell, a second moiety which promotes the internalization of the conjugate by the cell, and a third moiety comprising a polyaminoacid carrier and one or more cytotoxic molecules attached to said carrier.

37. The composition of claim 1, further comprising a substance which promotes the internalization of the conjugate.
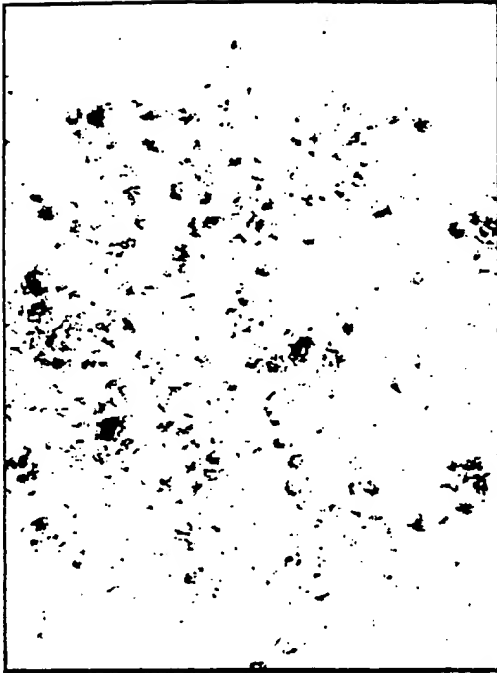
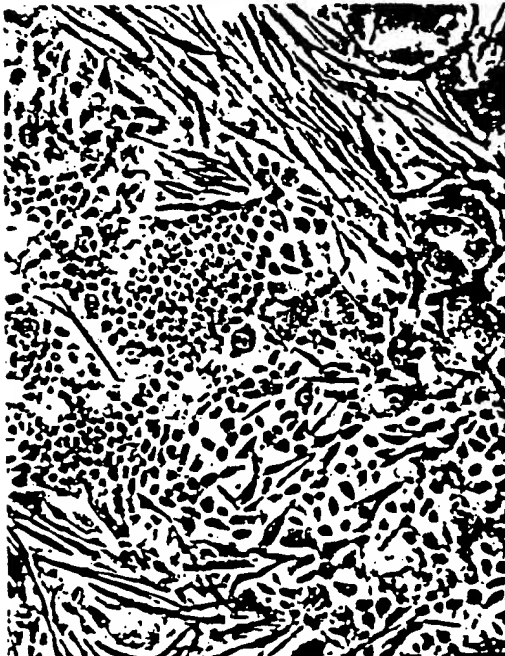38. The composition of claim 1, further comprising a mitogenic substance.

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FIG. 1a

FIG. 1b

FIG. 2a

FIG. 2b

FIG. 3a

FIG. 3b

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PARTIAL EUROPEAN SEARCH REPORT
which under Rule 45 of the European Patent Convention
shall be considered, for the purposes of subsequent
proceedings, as the European search report

Application number

EP 87 20 1490

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
X,Y	EP - A - 0 094 844 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA) * Page 13, line 10 - page 15, lines 23-30; claims 1,12 *	1-5, 8-19, 21,22, 24,36 -38	A 61 K 47/00
Y	EP - A - 0 040 506 (TEIJIN LTD) * Page 3, line 3 - page 6, line 5; page 6, lines 8-14; pages 17-19 *	1-5, 8,9, 11-19, 24,36 -38	
Y	EP - A - 0 169 112 (SANOFI) * Page 1, lines 3-8; page 2, line 24 - page 3, line 20; page 4, lines 15-21 *	1-5, 8-11, 22,36 -38	TECHNICAL FIELDS SEARCHED (Int. Cl.4) A 61 K

INCOMPLETE SEARCH   -2-

The Search Division considers that the present European patent application does not comply with the provisions of the European Patent Convention to such an extent that it is not possible to carry out a meaningful search into the state of the art on the basis of some of the claims.

Claims searched completely:

Claims searched incompletely:

Claims not searched: 25-35

Reason for the limitation of the search:

Method for treatment of the human or
animal body by surgery or therapy
(see art. 52(4) of the European Patent
Convention).

Place of search	Date of completion of the search	Examiner
The Hague	02-12-1987	BERTE

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
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A : technological background
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T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
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	DOCUMENTS CONSIDERED TO BE RELEVANT		CLASSIFICATION OF THE APPLICATION (Int. Cl.4)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
D,P X	JOURNAL OF CONTROLLED RELEASE, vol. 2, 1985, pages 205-213 Elsevier Science Publishers B.V. Amsterdam, NL; T. HOES et al.: "Optimization of macromolecular prodrugs of the antitumor antibiotic adriamycin" * Figures 2,3 *	1-5, 15,36-38	
Y	CHEMICAL ABSTRACTS, vol. 102, no. 13, 1st April 1985, ref.no. 105873q, Columbus, Ohio, US; B. LESUR et al.: "Covalent linkage of anthracyclines to macromolecular carriers", & PROTIDES BIOL.FLUIDS 1984 (Pub. 1985), 32, 437-40 * Abstract *	1-5,9, 15,36-38	TECHNICAL FIELDS SEARCHED (Int. Cl.4)
Y	US - A - 4 522 750 (E.W. ADES) * Column 4, line 68 - column 5, line 60; claims 1,4,8,10 *	1-5,8-9,17-18,36-38	

EPO Form 1505.3 06.78